Positive association between serum silicon levels and bone mineral density in female rats following oral silicon supplementation with monomethylsilanetriol

Ravin Jugdaohsingh[a,*], Abigail Watson[a,b], Pinaki Bhattacharya[c], G Harry van Lenthe[c] and Jonathan J Powell[a]

[a]MRC Human Nutrition Research, Elsie Widdowson Laboratory, Cambridge, United Kingdom.

[b]School of Sport and Exercise Health Sciences, Loughborough University, Loughborough, LE11 3TU, UK.

[c]Department of Mechanical Engineering, KU Leuven, Heverlee, Belgium.

*Address correspondence to: Ravin Jugdaohsingh, MRC Human Nutrition Research, Elsie Widdowson Laboratory, Fulbourn Road, Cambridge CB1 9NL, UK; Tel: (+44) 1223 426356; Fax: (+44) 1223 437515; Email: ravin.jugdaohsingh@mrc-hnr.cam.ac.uk

Supported by: LLR-G5 Ltd (Castlebar, Ireland) and Medical Research Council (grant number MC_US_A090_0008/Unit Programme number U1059).

Supplementary data (Supplemental Tables 1-6 and Figures 1-3) is included with this submission.

Abstract

Purpose. Epidemiological studies report strong positive associations between dietary silicon (Si) intake and bone mineral density (BMD) in pre-menopausal women and indicate that the association may be mediated by estradiol. We have tested this possibility in a mixed gender rodent intervention study.

Methods. Tissue samples were obtained from three groups of 20-week old Sprague Dawley rats (five males and five females per group) that had been supplemented *ad libitum* for 90 days in their drinking water with (i) < 0.1 mg Si/L (vehicle control) (ii) 115 mg Si/L (moderate dose) or (iii) 575 mg Si/L (high dose). All rats received conventional laboratory feed, whilst supplemental Si was in the form of monomethylsilanetriol, increasing dietary Si intakes by 18 and 99%, for the moderate and high dose groups, respectively.

Results. Fasting serum and tissue Si concentrations were increased with Si supplementation ($p < 0.05$), regardless of gender. However, only for female rats was there (i) a trend for a dose responsive increase in serum osteocalcin concentration with Si intervention and (ii) strong significant associations between serum Si concentrations and measures of bone quality ($p < 0.01$). Correlations were weaker or insignificant for tibia Si levels and absent for other serum or tibia elemental concentrations and bone quality measure.

Conclusions. Our findings support the epidemiological observations that dietary Si positively impacts BMD in younger females, and this may be due to a Si-estradiol interaction. Moreover, these data suggest that the Si effect is mediated systemically, rather than through its incorporation into bone.

Keywords: Animal study; Bone μCT; Estradiol; Matrix mineralisation; Nutrition; Silicon

Mini abstract

Observational (epidemiological) studies suggest the positive association between dietary silicon intake and bone mineral density may be mediated by circulating estradiol level. Here we report the results of a silicon supplementation study in rats that strongly support these observations and suggest an interaction between silicon and estradiol.

Introduction

Silicon, a major component of the mammalian diet via the consumption of plant-based foods, is present in all mammalian tissues and especially the connective tissues [1,2]. However, it is not clear whether it has a biological/biochemical role in higher animals, including mammals. Evidence amassed over the past 40 years suggests that Si may be important for normal bone and connective tissue health [1]. We have previously reported, in the Framingham Offspring cohort, that higher intakes of dietary Si are associated with higher BMD at the hip sites in pre-menopausal women and to some extent in men but not at all in post-menopausal women [3]. These findings suggested that there may be an interaction between Si intake and estrogen status and this was investigated further in a female-only cohort (the Aberdeen Prospective Osteoporosis Screening Study) where post-menopausal use of hormone replacement therapy (HRT) was documented in detail. We confirmed the Si-BMD relationship in pre-menopausal women and the lack of association in post-menopausal women who had never taken HRT [4]. However, for post-menopausal women, the Si-BMD relationship was regained in past users of HRT and especially so in current users of HRT [4]. These findings, from observation studies, imply a possible interaction between Si intake and estrogen status.

Others have also suggested a possible interaction between silicon and estrogen. Charnot & Peres [5,6] reported that endogenous sex and endocrine hormones affect the absorption and metabolism of Si in rats, while Nielsen & Poellot [7] reported that dietary Si (or Si status) affects the response to a change in estrogen status (i.e. ovariectomy/estrogen deficiency). Here we have taken advantage of rat tissue samples that were collected from a 12 week (90 day) oral intervention study with the Si supplement 'monomethylsilanetriol' (MMST, $CH_3Si(OH)_3$) to directly investigate the interaction between Si intake and oestrogen status with respect to bone health. The effect of Si supplementation on body Si pools (Si status) was investigated by measuring fasting Si levels in serum, ear (non-calcified collagenous tissue and

potential Si pool) and bone (calcified collagenous tissue and Si pool). The study was carried out by a commercial clinical research organisation for separate, regulatory purposes (i.e. a safety study), but it provided an opportunity for us to investigate the effects of three months Si supplementation on bone quality (bone microarchitecture and bone mineralisation) in male and female rats, where there is natural separation of circulating estradiol levels [8,9]. Silicon supplementation was given on a normal dietary Si background; i.e. this was not a deficiency study, the rats received a maintenance diet with its normal high Si content.

Previous human studies have shown, over a one month intervention period, that MMST ($CH_3Si(OH)_3$) is a safe Si supplement and that it undergoes metabolism to orthosilicic acid (OSA, $Si(OH)_4$) which is considered the bioactive form of Si [10,11]. Unlike OSA, however, this MMST precursor form has the advantage of remaining soluble and bioavailable at the supplemental levels used in this study [10-13].

Methods

Animal study and tissue collection

Rat tissue samples were collected at the end of a 90-day supplementation study with MMST, which was performed at a Good Laboratory Practice-accredited commercial clinical research organisation (CRO; Charles River Laboratories Pre-Clinical Services, Ireland). The study consisted of three groups each of ten rats: Group 1 = vehicle control (reverse osmosis treated drinking water with < 0.1 mg Si/L), Group 2 = 115 mg Si/L drinking water ('moderate Si dose') and Group 3 = 575 mg Si/L drinking water ('high Si dose'). The drinking waters were prepared and provided by LLR-G5 Ltd (Castlebar, Ireland), with Si supplemented in the form of MMST ($CH_3Si(OH)_3$), and Si contents were confirmed in our laboratory. The supplemental dosing undertaken in this study was, primarily, for regulatory safety assessment purposes and, therefore, the doses were high. Each group consisted of five male (8 weeks old at start) and five female (8 weeks old at start and nulliparous and non-pregnant) Sprague Dawley rats (Charles River Laboratories, Margate, UK). All rats were individually housed in polypropylene cages with stainless steel lids (with dust-free sawdust as bedding) at 22 °C with a 12 h/12 h light/dark cycle. The drinking water, with and without the Si supplementation, was provided *ad libitum* in individual (dedicated) plastic drinking units. All rats also received *ad libitum* the same maintenance feed (2018 Teklad 18% Protein Rodent diet).

Body weight and the consumption of the drinking waters were monitored on a daily basis, but feed intakes were not monitored. On day 89, the animals were fasted except for de-ionised water with no added Si. Fifteen to sixteen hours later, on day 90, animals were anesthetised, terminal blood samples were collected, animals euthanised and necropsy performed. Fasting blood, sera, plasma and tissue samples were generated for clinical biochemistry analysis, haematological analysis, and histopathology at the CRO and were not avalable to the authors for the below analyses. In addition, sera (obtained without use of anti-

coagulants), ears and tibias were also collected and stored at -80°C prior to being couriered frozen on dry ice to the authors' laboratory for analysis. This study was approved by the animal Ethics committee of the CRO.

Analyses

A brief summary of the analyses is given below with more details in Online Supporting Materials. All samples were analysed in a blinded fashion.

Serum 17β-estradiol

Fasting serum samples were analysed for 17β-estradiol using a commercially available high sensitivity ELISA kit (Enzo Life Sciences UK Ltd, Exeter, UK) to confirm the higher circulating serum levels in female rats compared to male rats and to investigate potential changes in circulating levels with Si treatment.

Serum osteocalcin

Aliquots of fasting serum samples were also analysed for osteocalcin, a marker of bone formation/bone turnover, using the commercially available Rat N-Mid Osteocalcin kit (MyBioSource Inc, Sand Diego, USA).

Total elemental analysis

Total elemental analyses of the rodent feed, fasting serum samples and, one of the pairs of ear and tibia samples were carried out by inductively coupled plasma optical emission spectrometry (ICP-OES), Jobin Yvon 2000-2 (Instrument SA, Longjumeau, France), using peak profiles [12,14] and sample-based standards for Si and other elements. Serum iron and phosphorus were not measured, the latter due to possible haemolysis [15].

Bone quality measurements

The second of the pair of tibias collected from each animal at necropsy was cleaned as previously described [16] at the authors' laboratory and couriered, frozen on dry ice, from the authors' laboratory to the Laboratory of Pathophysiology, University of Antwerp, Belgium for micro-CT analysis (Skyscan 1076 *in-vivo* X-ray micro-CT scanner, Aartselaar, Belgium). The following bone quality parameters (measures) were obtained: trabecular BMD (tBMD), tissue volume (TV), bone volume (BV), bone volume fraction (BV/TV), total surface (TS), bone surface (BS), bone surface/volume ratio (BS/BV), bone surface density (BS/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N) and total porosity (Po(T)).

Biomechanical testing

Following bone quality measures, the tibias were subjected to three-point bending test at room temperature in a custom made loading device, integrated in a materials testing machine (Bose ElectroForce Test Bench LM1, Bose Corp, USA). The following parameters were obtained from the data collected: stiffness (k; N/mm), yield strength (F_y; N) and fracture load (F_{max}; N).

X-ray diffraction

One tibia from each of the three groups of the female rats that had undergone micro-CT analysis underwent powder XRD analysis at the Institute for Materials Research, University of Leeds (UK) to determine changes in the mineral phase with Si supplementation. Prior to analysis the bones were processed [17] to remove organic components from the bone matrix that could interfere with the XRD analysis.

Statistical analyses

Results are reported as means ± SD unless otherwise stated. Grubbs' test for outliers was carried out (in GraphPad Prism 6; GraphPad Software Inc, La Jolla, US) on all the datasets collected. One of the serum Si values (1,120 µg/L), in a female rat from the 'high' Si dose group, was identified as a significant outlier (at $p < 0.05$) and is omitted from the data shown. Test for linearity was used to test for a dose-responsive increase in bone, serum and ear Si concentrations and serum osteocalcin concentration with Si supplementation and significance was taken as $p \leq 0.05$. In the absence of a significant trend, individual group differences between Si treatments and diluent control were then assessed by independent (unpaired) Samples 2-Tailed T-test and because there was comparison for two groups (moderate and high Si dosing) a Bonferroni correction was applied to the p value (i.e. p/n), and significance was taken as $p \leq 0.025$. Pearson correlation (with 2-Tailed T-test) was used to test for correlations between fasting serum and tibia element concentrations with bone quality measures. All statistical analysis was conducted in IBM SPSS version 21 (IBM Corporation).

Results

Serum 17β-estradiol concentrations

Fasting serum levels of 17β-estradiol, in the samples collected at necropsy, were, on average, 1.7-fold higher in female rats compared to male rats (94.8 ± 20.6 vs 57.9 ± 12.6 pg/ml; $p = 0.0002$; Supplementary Figure 1).

Silicon intakes and body weights

The feed (chow pellets) consumed by all groups contained, on average, 610 µg Si/g feed (Supplementary Table 1) and, assuming typical average feed intakes of 23 g feed/day in adult female rats and 32 g feed/day in adult males rats of the same strain [18], this would contribute ~ 14 and 19 mg Si to daily Si intakes, respectively. Mean daily intake of Si from drinking waters (supplemented with and without Si) are shown in Table 1, separately for male and female rats, and did not differ between genders: overall mean Si intakes from the moderate and high Si-supplemented drinking waters added a further 18 and 99% Si to that from the feed.

Weight gain of the animals was unaffected by Si supplementation (Supplemental Figure 2; $p > 0.2$ for female rats and $p > 0.4$ for male rats). There was also no association between serum Si and body weight or body weight gain ($r = -0.2$, $p > 0.5$). Moreover, no adverse effects (clinically, biochemically or pathologically) were associated with three months' MMST supplementation at either the moderate or high doses investigated (data not shown), consistent with previous findings in a lower dose, 4-week human supplementation study [10].

Tissue silicon measurements

A dose-responsive increase in Si concentrations of the serum and collagen-rich ear tissue was apparent with Si supplementation ($p < 0.05$, test for linearity; Figure 1a & 1b). However, for

the tibia, there was not a dose response: whilst the moderate dose of supplemental Si led to a significant increase in bone Si levels ($p = 0.03$) the high dose had no effect (Figure 1c). Similar patterns were observed for male and female rats (data not shown).

Bone associated elements

Serum Cu and Zn concentrations were significantly increased in female rats on moderate dose Si supplementation and a similar, but non-significant, trend was seen for tibia Cu and Zn levels (Supplemental Tables 2 & 3). Male rats showed a similar trend for serum and tibia Cu levels (Supplemental Tables 2 & 3). Moreover, in the female rats, tibia Ca and tibia Ca:P ratios were increased with the high dose of Si compared to controls ($p = 0.025$ and 0.016 respectively; Figure 2), while in male rats no statistically significant differences in tibia concentrations of Ca, P or Ca:P were found with Si supplementation ($p > 0.1$ compared to controls; data not shown).

Serum Osteocalcin

Serum osteocalcin concentrations were similar in female and males rats ($p = 0.45$) but in female rats there was a trend for a dose dependent increase in osteocalcin concentration with Si supplementation (Figure 3). Moreover, although serum osteocalcin concentrations showed no correlation with serum Si concentrations in female rats ($p = 0.3$), strong *negative* correlation was seen in male rats ($r = -0.72$, $p = 0.008$).

Bone quality measures

Silicon supplementation had no effect on tBMD in male rats (Figure 2d & Supplemental Table 4) and, although mean tBMD increased for female rats, it was not significant either (Figure 2D & Supplemental Table 4). Nonetheless, given the relatively low group numbers

and the variance around serum and bone Si levels, we considered that an association between Si levels and tBMD could have been masked by categorical analysis. To this end direct correlations showed a strong relationship between fasting serum Si levels (a recognised proxy for Si status (10)) and tBMD in female rats but not in male rats (Figure 4a vs. 4b). Bone Si levels also correlated with tBMD for female rats only (Figure 4c vs. 4d), albeit not as strongly as between serum Si and BMD and perhaps explained by the association between serum Si and bone Si levels (Figure 4e & 4f).

Moreover, fasting serum Si concentrations were found to correlate strongly with other bone quality measures and, again, only for the female rats. Associations were positive with BV/TV, BS/TV and with Tb.N, and negative with Tb.Sp and Po(T) (Figure 5). Strong positive correlations were also obtained for fasting serum Si levels and, individually, BV ($r = 0.79$, $p = 0.0008$) and BS ($r = 0.72$, $p = 0.004$) but not TV ($r = -0.06$, $p = 0.835$) or TS ($r = 0.02$, $p = 0.934$). So serum Si in female rats was associated with the amount of bone, but not the size of bone. A weaker (positive) correlation was obtained with Tb.Th ($r = 0.64$, $p = 0.014$).

The above correlations were also generally found with tibia Si levels but, again, were not as significant as for the fasting serum Si levels (data not shown). In the single indicator tibia samples taken from each group for *ex-vivo* X-ray diffraction analyses there was no suggestion that Si supplementation altered bone mineral phase (Supplemental Figure 3). Silicon intervention did not significantly altered bone strength based upon categorical analysis (Supplemental Table 5) whilst, unlike for tBMD, the positive correlation with serum Si was not significant (Figure 5f-h).

Finally, to address the specificity of silicon's association with bone quality, we next assessed correlations of other serum ($n = 5$) and tibia ($n = 8$) elemental concentrations with tBMD (see methods). Of these only serum Mg yielded a (weak) correlation with tBMD ($r =$

0.64, p = 0.015 in female rats and r = 0.61, p = 0.016 in male rats), but this did not carry through with any other bone quality measures (data not shown).

It should be noted that in female rats, serum estradiol showed no correlations with serum Si, tibia Si, serum osteocalcin or tBMD (Supplemental Table 6). A significant correlation was obtained with TbTh (r = -0.67, p = 0.05; data not shown), but this was in the opposing direction to serum Si. In male rats serum estradiol showed no association with bone quality measures, although significant association with serum osteocalcin (r = 0.71, p = 0.033) and Fy (r = -0.88, p = 0.004) were obtained (Supplemental Table 6).

Discussion

As noted above we have previously reported, in human epidemiological studies, a strong positive association between dietary Si intake and BMD in pre-menopausal women [3,4], whilst the lack of association in post-menopausal women was restored for those taking hormone replacement therapy [4]. We thus proposed that the dietary Si-BMD effect is estradiol mediated [3]. Assuming that Si has some active beneficial role in human and other mammalian connective tissues then these prior studies [3,4], and other data [1], indicate that the chemical species responsible is almost certainly orthosilicic acid ($Si(OH)_4$). Dietary Si appears to be absorbed only in monomeric form from the gastrointestinal tract [12,19], either directly so from fluids such as drinking water, or, following digestion of plant-based foods. For these reasons the CRO-based three month supplementation study that is described herein provided an excellent opportunity to test the hypothesis that dietary Si positively impacts BMD in estradiol-replete mammals. Firstly, unlike orthosilicic acid which starts to form insoluble silicates much above 56 mg Si/L (2 mM Si), MMST ($CH_3Si(OH)_3$) may be added to drinking water at up to 588 mg Si/L (21 mM Si) without irreversible polymerisation and precipitation. Moreover, MMST appears entirely non-toxic, again as confirmed herein, and is metabolised to orthosilicic acid *in vivo* [10]. Secondly, in murine models a three-month time period should be sufficient time to see the impact on BMD of effective intervention [20]. Thirdly, male and female rats differ in their circulating estradiol levels by 1.7 fold in this study and by even greater amounts in prior studies [8,9].

Fasting serum concentrations of Si provide the best known measure of Si status because recently ingested and absorbed Si is rapidly cleared from the circulation and, hence, fasting levels provide a steady state measure of Si that is presumed to be in equilibrium with body stores [10]. The finding that, following intervention, fasting serum Si levels were strongly positively correlated with trabecular BMD in female rats but not male rats supports the

hypothesis that estradiol is required for the *in vivo* beneficial utilisation of Si. Of course other hormonal differences between male and female rats (i.e. other than estradiol) may explain or contribute to these findings. However, a previous study that looked at the effects/contribution of the different sex and endocrine hormones on the absorption of Si and tissue Si levels in adult rats found that estrogen deficiency in female and male rats produced the most pronounced effects [5], suggesting that estradiol may be the main or most potent mediator of Si metabolism. Similarly, with regards to bone, estrogen deficiency has the most marked effect on bone growth in male and female rats [21]. Replication of our findings in intact (sham-operated) and estradiol–supplemented ovariectomised rats but not in ovariectomised rats would provide the best proof for this, because, as mentioned above, in our previous observational study, the Si-BMD relationship was regained in post-menopausal women who were taking HRT [4].

Whether there is a small effect of oral Si on tBMD in male rats, as is observed for dietary Si–BMD associations in male humans [3], would probably require greater study numbers for intervention than we had access to in this work. It is also possible that in male rats Si supplementation affects a different bone compartment: i.e. cortical bone rather than (or more than) trabecular bone. Cortical bone thickness was not measured in this study and biomechanical data (which mainly evaluates cortical bone properties; see below) was incomplete for male rats (Supplemental Table 5). To our knowledge the effects of Si supplementation on cortical and trabecular bone compartments have not been directly evaluated in the literature even though, our previous epidemiological study showed similar Si-BMD associations at the different hip sites and the lumbar spine in men [3], implying that Si may affect both bone compartments equally.

How dietary Si could promote BMD in ‘estradiol-replete’ mammals is presently unclear, although additional observations herein may provide some clues. For example, tibia

Si levels showed some correlation with tBMD and other bone quality measures but these were either not significant or were weak compared to the serum Si correlations with BMD. This suggests that the Si effect is not due to and/or sensed from direct incorporation of Si into bone but, rather, is a peripherally-generated signal as previously argued [16]. Indeed, although only three bone samples were analysed by XRD, there was certainly no obvious change to bone mineral with Si supplementation. This is not surprising, as the highest increase in bone Si content, with Si supplementation, was < 0.01 atomic mole % and thus unlikely to directly affect the mineral phase or its properties. In fact, tibia Si levels did not increase linearly with Si supplementation (Figure 1). This was not a result of the higher dose being less bioavailable, as indicated by the increase in fasting serum and ear tissue Si levels compared to the moderate Si dose groups. It is more likely that it indicates a safety mechanism: a negative feedback to protect against marked changes in bone composition and/or over mineralisation, which could affect bone quality and bone strength, as suggested by Reffitt *et al*. [22] and consistent with our more recent data [16,23]. Together, these findings suggest that different tissues could have differing Si tolerances/requirements and, in bone, this may have been surpassed with the high Si dose albeit not for the collagenous ear tissue. However, to confirm this, additional doses of Si/MMST should be tested.

The specific strong correlations between serum Si concentrations and bone quality measures, and the lack of similar correlations between serum estradiol with either bone quality measures or with serum Si concentrations, suggest that the effect of Si is not directly through estradiol or changes in estradiol concentrations. As such the findings suggest that estradiol mediates the effect of Si rather than *vice versa*.

The positive association between fasting serum Si concentration and tBMD in female rats was backed up by strong correlations with other bone quality measures (Figure 5) and the trend for a dose-dependent increase in serum osteocalcin concentration. Overall serum Si in

the female rats correlated positively with the amount of bone tissue (BV, BS, BV/TV, BS/TV, Tb.Th and Tb.N) and negatively with the amount of non-bone tissue/space (Tb.Sp and Po(T)), i.e. suggesting that Si supplementation is associated with increased bone tissue within the volume measured. These findings did not, however, proceed to a correspondingly significant increase in bone strength. There are two possible explanations. Firstly, it is possible for BMD to be increased without an increase in bone strength/bone stiffness. For example, the addition of bone to the endocortical surface of female rats does not lead to an increase in bone strength [21]. Female rats have higher BMD compared to male rats, but this is not associated with higher bone stiffness/bone strength and is in fact associated with lower bone stiffness/bone strength than male rats ([21]; Supplementary Tables 4 & 5). The second possibility is that Si affects trabecular bone (and therefore tBMD) but not cortical bone in female rats. The three-point bending test evaluates the shaft of the bone, i.e. cortical bone properties (e.g. cortical thickness and cross-sectional area). Hence, it is possible that Si could change bone micro-architecture without effects on bone stiffness as assessed by three-point bending. The lack of correlation between tBMD and bone strength measures here supports this statement (data not shown).

Finally, these data also show specificity in the association with tBMD to Si as the other serum and tibia elements investigated (including Cu, Zn, Mg, Ca, P and Ca:P ratio), either showed no correlation with bone quality measures or were markedly weaker (data not presented), regardless of gender. In female rats the weak correlations observed for serum Mg concentrations with tBMD ($r = 0.64$, $p = 0.015$), BS/TV ($r = 0.53$, $p = 0.051$), TbN ($r = 0.53$, $p = 0.053$), and Po(T) ($r = -0.55$, $p = 0.044$), and for serum P concentrations with BV/TV ($r = 0.54$, $p = 0.046$) and Po(T) ($r = -0.54$, $p = 0.046$), are most likely driven by their associations with serum Si concentrations ($r = 0.63$, $p = 0.016$ for serum Mg and $r = 0.57$, $p = 0.035$ for serum P). Silicon supplementation increased serum and tibia Cu concentrations in both male

and female rats, and serum and tibia Zn concentrations in female rats. Similar findings have previously been reported. Emerick & Kayongo-Male [24] reported that Si supplementation increased the Cu status (plasma Cu concentrations) of both Cu-deplete and Cu-replete rats while, more recently, Seaborn & Nielsen [25] reported that Si deprivation reduced femoral and vertebral rat bone Cu and Zn concentrations. Emerick & Kayongo-Male [24] went further to suggest that some of the reported effects of Si (on connective tissues) may be attributed to an increase in Cu utilisation. However, as noted above, we did not find any correlations between serum or tibia Cu concentrations with bone quality measures, but we did with serum Si, suggesting that, at least here, the Si effect on bone quality was not driven by the increase in Cu utilisation.

Previous studies have shown that when the bone steady-state (equilibrium) is challenged, such as with ovariectomy, osteopenia or reproduction, oral or intravenous Si intervention can help maintain BMD [26-31] (see also reviews by Jugdaohsingh [1] and Price *et al*. [32]). In the work presented here, however, the rats were healthy. Moreover, the rats were not Si deficient so the effects seen are not the correction of a state of stress but, rather, are offering insights into 'optimal nutrition'. The supplemental dosing undertaken in this study was, primarily, for regulatory safety assessment purposes and, therefore, the doses were high. In the 'moderate' dose group, 115 mg Si/L (4.1 mM MMST) was the sole source of fluid. In adult human supplementation it would be just 90 mL/day out of, typically, 2 L total fluid intake per day [10]. The 'high' dose group was the same except the Si concentration was 575 mg Si/L (20.5 mM MMST) instead of 115 mg/L. Translating these findings to human intakes of Si is not easy. On the one hand, as noted above, supplementation in the rats was disproportionately high compared to human dosing. On the other hand, nutrient intakes are always disproportionately high for rats versus humans [33] and the Si supplementation of this study only increased the rats naturally high dietary Si intake by 18.0% and 99% with

moderate and high dosing respectively. Interestingly, by analogy, the correlation between dietary Si intake and BMD in pre-menopausal women of the Framingham cohort [3] shows no tail-off in the relationship at the upper quintile of Si intake (30-63 mg/day), so perhaps optimal dietary Si intakes in pre-menopausal women could indeed be higher.

Conclusion

In conclusion, this paper reports that Si supplementation increases fasting serum and connective tissue Si concentrations in rats. In female rats, concentrations of serum Si, but not other bone or serum elements, correlated strongly with trabecular BMD and other bone quality measures. These relationships were not seen in male rats and were not seen with measures of soft tissue quality for either gender, supporting the hypothesis that estradiol is required for the optimal utilisation of dietary Si in bone/connective tissues. However, additional animal models (e.g. estrogen receptor knockouts (ER-null) or ovariectomy with and without estradiol) are required to confirm this. The effect appears to be related to systemic signalling, governed by steady state circulating Si levels, rather than direct incorporation of Si into bone. Further work should also aim to identify the mechanism.

Acknowledgements: We thank Dr Lorenzo Glorie and Prof Patrick D'Haese (Laboratory of Pathophysiology, University of Antwerp) for the micro-CT analysis, Drs Tim Comyn and Andrew Brown (Institute for Materials Research, University of Leeds) for the XRD analysis, Ms Jennifer Gatzemeier and Ms Marika Mancino for help with sample preparation and data collation and Dr Kate Ward for useful discussion.

The authors' laboratories have received research funding from the silicon supplement and food industry; for the work presented here the animal study from which the tissues were collected and costs of the micro-CT analysis of the tibias were provided by LLR-G5 Ltd (Castlebar, Ireland). All remaining costs were met through core institutional funds: Medical Research Council (grant number MC_US_A090_0008/Unit Programme number U1059). The research was designed, executed, analysed and communicated only by the authors.

Conflict of Interest: JJP has consulted to companies involved with silicon supplementation including LLR-G5 Ltd. All authors declare no conflict of interest.

Authors' contributions: R. J. and J. J. P. designed the research in conjunction with the CRO; R. J. and A. W. conducted the research; P. B. and G. H. L. undertook the biomechanical testing; R. J. had study oversight; R. J. and J. J. P. analysed the data; R. J. and J. J. P. wrote the paper and had primary responsibility for final content. All authors read and approved the final manuscript.

Ethical approval: All applicable institutional and/or national guidelines for the care and use of animals were followed.

References

[1] Jugdaohsingh R (2007) Silicon and bone health. J Nutr Health Aging 11:99-110.

[2] Carlisle EM (1986) Silicon as an essential trace element in animal nutrition. In: Silicon Biochemistry, Ciba Foundation Symposium 121. John Wiley and Sons Ltd, Chichester, pp 123-139.

[3] Jugdaohsingh R, Tucker KL, Qiao N, Cupples LA, Kiel DP, Powell JJ (2004) Dietary silicon intake is positively associated with bone mineral density in men and premenopausal women of the Framingham Offspring Cohort. J Bone Miner Res 19:297–307.

[4] Macdonald HM, Hardcastle AC, Jugdaohsingh R, Fraser WD, Reid DM, Powell JJ (2012) Dietary silicon interacts with oestrogen to influence bone health: evidence from the Aberdeen Prospective Osteoporosis Screening Study. Bone 50:681-87.

[5] Charnot Y, Pérès G (1971) Change in the absorption and tissue metabolism of silicon in relation to age, sex and various endocrine glands. Lyon Medicine 226:85-88.

[6] Charnot Y, Asseko MC, Peres G (1977) Comparison of effects of ingestion of Si on the content of Ca and Mg of different tissues of female rats normal or receiving oral oestrogen-gestogens. Ann Endocrinol (Paris) 38:377-378.

[7] Nielsen FH, Poellot R (2004) Dietary silicon affects bone turnover differently in ovariectomized and sham-operated growing rats. J Trace Elem Exp Med 17:137-149.

[8] Zhong P, Lui XY (2011) Relationship between the levels of sex hormones and loss of bone mass in aging male rats. Zhonghua Nan Ke Xue 17:717-21.

[9] Sokol RZ, Okuda H, Stanczyk FZ, Wolfe GW, Delaney JC, Chapin RE (1999) Normative reproductive indices for male and female adult Sprague-Dawley rats. Contraception 59:203-207.

[10] Jugdaohsingh R, Hui M, Anderson SHC, Kinrade SD, Powell JJ (2013) The silicon supplement monomethylsilanetriol is safe and increases the body pool of silicon in healthy pre-menopausal women. Nutr Metab (Lond) 10:37.

[11] Jugdaohsingh R, Anderson SHC, Kinrade SD, Powell JJ (2013) Response to Prof. D. Vanden Berghe letter: 'There is not enough data to conclude that monomethylsilanetriol is safe' Nutr Metab (Lond) 10:65.

[12] Sripanyakorn S, Jugdaohsingh R, Dissayabutr W, Anderson SHC, Thompson RPT, Powell JJ (2009) The comparative absorption of silicon from different foods and food supplements. Br J Nutr 102:825-34.

[13] Allain P, Cailleux A, Mauras Y, Renier JC (1983) Digestive absorption of silicon after a single administration in man in the form of methylsilanetriol salicylate. Therapie 38:171-174.

[14] Burden TR, Powell JJ, Thompson RPH, Taylor PD (1995) Optimal accuracy, precision and sensitivity of inductive coupled plasma optical emission spectrometry: bioanalysis of aluminium. J Anal At Spectrom 10:259-66.

[15] Reffitt DM, Burden TJ, Seed PT, Wood J, Thompson RP, Powell JJ (2000) Assessment of iron absorption from ferric trimaltol. Ann Clin Biochem 37:457-66.

[16] Jugdaohsingh R, Calomme MR, Robinson K, Nielsen F, Anderson SH, D'Haese P, Geusens P, Loveridge N, Thompson RP, Powell JJ (2008) Increased longitudinal growth in rats on a silicon-depleted diet. Bone 43:596-606.

[17] Kim HM, Rey C, Glimcher MJ (1995) Isolation of calcium-phosphate crystals of bone by non-aqueous methods at low temperature. J Bone Miner Res 10:1589-601.

[18] Hubert MF, Laroque P, Gillet J-P, Keenan KP (2000). The effects of diet, ad libitum feeding, and moderate and severe dietary restriction on body weight, survival, clinical

pathology parameters, and cause of death in control Sprague-Dawley rats. Tox Sci 58:195-207.

[19] Jugdaohsingh R, Reffitt DM, Oldham C, Day JP, Fifield LK, Thompson RPH, Powell JJ (2000) Oligomeric but not monomeric silica prevents aluminum absorption in humans. Am J Clin Nutr 71: 944-49.

[20] Halloran BP, Wronski TJ, VonHerzen DC, Chu V, Xia X, Pingel JE, Williams AA, Smith BJ (2010) Dietary dried plum increases bone mass in adult and aged male mice. J Nutr 140:1781-87.

[21] Callewaert F, Venken K, Kopchick JJ, Torcasio A, van Lenthe GH, Boonen S, Vanderschueren D (2010) Sexual dimorphism in cortical bone size and strength but not density is determined by independent and time-specific actions of sex steroids and IGF-1: evidence from pubertal mouse models. J. Bone Min. Res 25:617-626.

[22] Reffitt DM, Ogston N, Jugdaohsingh R, Cheung HF, Evans BA, Thompson RP, Powell JJ, Hampson GN (2003) Orthosilicic acid stimulates collagen type 1 synthesis and osteoblastic differentiation in human osteoblast-like cells in vitro. Bone 32:127–35.

[23] Jugdaohsingh R, Anderson SHC, Calomme MR, Robinson K, Nielsen F, Anderson SH, D'Haese P, Geusens P, Loveridge N, Thompson RP, Powell JJ (2009) Letter to the editor. Reply to Dr Exley comments on: Jugdaohsingh R *et al.*, Increased longitudinal growth in rats on a silicon-depleted diet. Bone 44:1019-20.

[24] Emerick RJ, Kayongo-Male H (1990) Silicon facilitation of copper utilization in the rat. J Nutr Biochem 1:487-90.

[25] Seaborn CD, Nielsen FH (2002) Dietary silicon and arginine affect mineral element composition of rat femur and vertebra. Biol Trace Elem Res 89:239-50.

[26] Calomme M, Geusen P, Demeester N, Behets GJ, D'Haese P, Sindambiwe JB, Van Hoof V, Vanden Berghe D (2006) Partial prevention of long-term femoral bone loss in

aged ovariectomized rats supplemented with choline-stabilized orthosilicic acid. Calcified Tissue Int 78:227-32.

[27] Onderci M, Sahin N, Sahin K, Balci TA, Gursu MF, Juturu V, Kucuk O (2006) Dietary arginine silicate inositol complex during the late laying period of quail at different environmental temperatures. Br Poultry Sci 47:209-15.

[28] Bae YJ, Kim JY, Choi MK, Chung YS, Kim MH (2008) Short-term administration of water-soluble silicon improves mineral density of the femur and tibia in ovariectomized rats. Biol Trace Elem Res 124:157-63.

[29] Spector TD, Calomme MR, Anderson SH, Clement G, Bevan L, Demeester N, Swaminathan R, Jugdaohsingh R, Berghe DA, Powell JJ (2008) Choline-stabilized orthosilicic acid supplementation as an adjunct to calcium/vitamin D3 stimulates markers of bone formation in osteopenic females: a randomized, placebo-controlled trial. BMC Musculoskelet Disord 9:85.

[30] Kim MH, Bae YJ, Choi MK, Chung YS (2009) Silicon supplementation improves the bone mineral density of calcium-deficient ovariectomized rats by reducing bone resorption. Biol Trace Elem Res 128:239-47.

[31] Eisinger J, Clairet D (1993) Effects of silicon, fluoride, etidronate and magnesium on bone mineral density: a retrospective study. Magnesium Res 6:247-49.

[32] Price CT, Koval KJ, Langford JR (2013) Silicon: a review of its potential role in the prevention and treatment of post-menopausal osteoporosis. Int J Endocrinol 2013:316783.

[33] Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board on Agriculture, National Research Council (1995) Nutrient requirements of laboratory animals. 4th Revised Edition. Washington DC: National Academy Press.

Table 1.

Mean daily silicon intakes from drinking waters[a]

	Female rats			Male rats		
	Group 1: Control (n=5)	Group 2: 115 mg Si/L (n=5)	Group 3: 575 mg Si/L (n=5)	Group 1: Control (n=5)	Group 2: 115 mg Si/L (n=5)	Group 3: 575 mg Si/L (n=5)
Drinking water (ml/day)[b]	21.5 (2.2)	22.9 (3.9)	26.5 (3.1)[d]	29.9 (3.0)	29.0 (5.4)	29.4 (4.7)
Si intake from water (mg/d)[c]	< 0.002	2.64 (0.45)	15.21 (1.77)	< 0.002	3.33 (0.62)	16.86 (2.71)

[a] Means (± SD) calculated from daily measurements between days 40 and 50. Feed intake was not measured but was estimated to be 23 g/d in female rats and 32 g/day in male rats, contributing ~ 14 and 19 mg Si/d, respectively in female and male rats.

[b] A vehicle control (reverse osmosis treated drinking water with < 0.1 mg Si/L) or Si-supplemented drinking water (115 mg Si/L or 575 mg Si/L) was consumed by the animals *ad libitum* as a substitute for normal drinking water.

[c] There was no significant difference in Si intake between female and male rats.

[d] Intake was significantly greater than control ($p = 0.02$, unpaired T-test).

Figure legends

Figure 1. Fasting silicon concentrations in the tissues of rats (n=10 per treatment: 5 males and 5 females), in the control and Si-supplemented groups, collected at necropsy (i.e. after 12 weeks intervention). Data is shown as box-plots where the horizontal lines indicate the 5^{th}, 25^{th}, 50^{th} (i.e. median), 75^{th} and 95^{th} percentiles, the open square shows the mean and the crosses the minimum and maximum values. Test for linearity was significant for serum Si ($p < 0.05$) and ear Si ($p < 0.0001$) concentrations, but not tibia Si concentration ($p = 0.17$). By subsequent t-test of the tibia, $p = 0.003$ for 115 mg Si/L vs. Control and was not significant for 575 mg Si/L versus control.

Figure 2. Calcium (**a**), phosphorus (**b**) and Ca:P (**c**) concentrations of the tibias of female rats (n=5 per treatment), in the control and Si-supplemented groups, at necropsy after 12 weeks supplementation. (**c**) Trabecular bone mineral density (tBMD) of the tibia of male and female rats at necropsy. Data is shown as box-plots where the horizontal lines indicate the 5^{th}, 25^{th}, 50^{th} (i.e. median), 75th and 95^{th} percentiles, the open square shows the mean and the crosses the minimum and maximum values. Tibia calcium concentration and Ca:P ratio were higher in the higher dose Si group, compared to the diluent control ($p = 0.025$, for the 575 mg Si/L vs. Control; unpaired T-test).

Figure 3. N-mid osteocalcin concentrations of the fasting serum, collected at necropsy, of the female (**a;** n=4 per treatment) and male (**b;** n=4-5 per treatment) rats, in the control and Si-supplemented groups after 12 weeks supplementation. Data is shown as box-plots where the horizontal lines indicate the 5^{th}, 25^{th}, 50^{th} (or median), 75^{th} and 95^{th} percentiles, the open square shows the mean and the crosses the minimum and maximum values.

Figure 4. (**a & b**) Associations between fasting serum silicon concentrations and trabecular bone mineral density (tBMD) of the tibias of female (**a**) and male (**b**) rats at necropsy (12 weeks intervention) (r= 0.90 & p < 0.0001 for the female rats; Pearson Correlation with 2-Tailed T-test). (**c & d**) Associations between tibia Si concentrations and tBMD in female (c) and male (d) rats at necropsy (r = 0.47 & p = 0.074 for the female rats; Pearson Correlation with 2-Tailed T-test). (**e & f**) Associations between fasting serum Si concentrations and tibia Si contents of the female (e) and male (f) rats at necropsy (r= 0.47 & p = 0.093 for the female rats; Pearson Correlation with 2-Tailed T-test). Note, the correlation reported in **a** was not dependent upon the serum Si value at 532 µg/L as its removal from the dataset only marginally affected the correlation reported; i.e. was still highly correlated (r = 0.8, p = 0.002).

Figure 5. Associations between fasting serum silicon concentrations and bone microarchitecture/quality of female rat tibias collected at necropsy (12 weeks intervention). Positive correlations were obtained for (**a**) bone volume fraction BV/TV (r = 0.90 & p < 0.0001; Pearson Correlation with 2-Tailed T-test), (**b**) bone surface density BS/TV (r = 0.91 & p < 0.0001; Pearson Correlation with 2-Tailed T-test), (**c**) trabecular number Tb.N (r = 0.90 & p < 0.0001; Pearson Correlation with 2-Tailed T-test), while negative correlations were with (**d**) trabecular separation Tb.Sp (r = -0.80 & p = 0.001; Pearson Correlation with 2-Tailed T-test) and (**e**) total porosity Po(T) (r= -0.90 & p < 0.0001; Pearson Correlation with 2-Tailed T-test). Correlations between fasting serum Si concentrations and bone stiffness (**f**), yield strength (**g**) and fracture load **(h)** were not significant; r = 0.15, 0.27 and 0.41 and p = 0.6, 0.4 and 0.1, respectively. The correlations reported are not dependent upon the serum Si value at 532 µg/L, as its removal from the dataset only marginally affected the correlations reported; i.e. all were still highly correlated (r = 0.8, p < 0.004).

Supplementary Data

Supplemental Materials and Methods

More thorough methodological details on the analyses performed in the paper are given below.

Materials

For sample analysis, ultra high purity (UHP) water was 18 MΩ/cm, from a Branstead Nano-Pure water purifier (Thermo Scientific; Ohio, USA). Nitric acid (69% (w/v) p.a. plus) was high purity from Fluka Ltd (Gillingham, UK). Hydrogen peroxide (30%, Suprapure), Si ICP stock standard solution (1000 mg/L Si), diethyl ether (AnalaR grade) and microcentrifuge tubes (1.5 mL) were from VWR Ltd (Poole, UK). Polypropylene (PP) tubes (13 mL & 50 mL) were from Sarstedt Ltd (Leicester, UK). Rat N-Mid Osteocalcin ELISA kit was from MyBioSource Inc (Sand Diego, USA). 17β-estradiol high sensitivity ELISA kit was from Enzo Life Sciences UK Ltd (Exeter, UK).

Assays

Serum 17β-estradiol

Fasting serum samples were analysed for 17β-estradiol to confirm the higher circulating serum levels in female rats compared to male rats and to investigate potential changes in circulating levels with Si treatment. Aliquots (150 µL) of the undiluted sera underwent liquid-liquid extraction with diethyl ether (750 µL) and then the extracts were air dried and reconstituted in 110 µL assay buffer (supplied in the ELISA kit). Samples were analysed using the 17β-Estradiol high sensitivity ELISA kit, following the kit's protocol. Absorbance was read at 405 nm using an optical microplate reader (LabSystems Multiskan RC).

Serum osteocalcin

Supplementary Data

The fasting serum samples were also analysed for osteocalcin, a marker of bone formation/bone turnover, using the commercially available Rat N-Mid Osteocalcin kit. Aliquots (100 µL) of the undiluted sera were used and the assay was carried out as per the instructions given in the kit protocol. Absorbance was read at 450 nm (detection wavelength) and 595 nm (correction wavelength) using an optical microplate reader (LabSystems Multiskan RC; Thermo Scientific, USA).

Total elemental analysis

Total elemental analyses were carried out by inductively coupled plasma optical emission spectrometry (ICP-OES), Jobin Yvon 2000-2 (Instrument SA, Longjumeau, France), equipped with a concentric nebuliser and cyclonic spray chamber. The sample flow rate was 1 mL/min. Peak profiles were used as previously described [12,14], with a window size of 0.08 nm (0.04 nm either side of the peak) with 21 increments per profile and an integration time of 0.5 second per increment. Analytical lines were: 251.611 nm (Si), 213.618 nm (phosphorus, P), 315.887 nm (calcium, Ca), 279.806 nm (magnesium, Mg), 766.490 nm (potassium, K), 213.856 nm (iron, Fe), 257.610 nm (zinc, Zn), 259.940 nm (manganese, Mn) and 324.754 nm (copper, Cu).

Accurate iron levels in serum are unreliable with this methodology due to lysed erythrocyte contributions of haem iron, which occurs even with invisible haemolysis [15]. Similarly, phosphate cannot be measured in this fashion as total serum phosphorus includes significantly more than just inorganic phosphate. Hence, these two elements were excluded from serum analysis but were maintained for bone analysis. All samples were analysed in a blinded fashion.

<u>Serum samples</u>

Supplementary Data

An aliquot of the blood samples collected with 20 gauge needle and syringe (5 & 10 mL) was collected into 13 mL polypropylene (PP) tube and allowed to clot at 4°C without anticoagulants. The clotted blood samples were centrifuged at 4°C in a refrigerated centrifuge (Labofuge 400R) and the separated serum collected in a clean 13 mL PP tube and stored at -80°C prior to being couriered on dry ice to the authors. Serum samples were analysed using methods previously described [16]. 1 mL aliquot was diluted 1+4 with 0.2% nitric acid (prepared by diluting 69% nitric acid in UHP water) in 13 mL PP tubes. After through mixing, 2 mL from each of the diluted sera samples was removed and pooled into a 50 mL PP centrifuge tube. Pooled sample based standards were prepared by spiking aliquots of the pooled diluted sera with Si (0-1 mg/L). Aqueous silicon standards (0-1 mg/L Si) were also prepared, in 0.2% nitric acid, to determine the Si content of the acid diluent. Diluted sera and acid diluent (0.2% HNO_3) were analysed with their appropriate set of standards in a single batch. For analysis of the other serum elements, the serum samples were diluted 50-fold in 0.2% nitric acid. Analysis was carried out in a blinded fashion, using appropriately prepared pooled, multi-element, sample-based standards (0-10 mg/L of each element). Serum manganese concentrations could not be assessed as concentrations in the diluted samples were not different to the acid diluent alone.

Ears

Ears were analysed for Si content as a source of non-articulating, non-calcified collagenous tissue and, thus, potential site of Si utilisation. One of the pair of ears from each animal was cleaned of excess hairs, washed in high-purity water, blotted dry and placed in pre-weighed 13 mL PP tubes. The mass was determined and then samples digested with 2.5 mL of a 1+1 mixture of concentrated nitric acid (69% pa plus) and 30% hydrogen peroxide. Sample blanks were similarly prepared. Samples and sample blanks were placed overnight in

a shaking incubator (HT Infors Minitron; Infors AG, Basel, Switzerland) set at 81 rpm and 45°C to digest. Upon complete digestion, the samples and sample blanks were diluted with 2 mL UHP water. One ml was then removed from each of the diluted samples and pooled in a 50 mL PP centrifuge tube. Pooled sample based standards were prepared by spiking aliquots of the pooled diluted sample with Si (0-10 mg/L). Aqueous acid-based Si standards (0-10 mg/L Si) were also prepared, in 19% nitric acid, to determine Si content of the sample blanks. The diluted samples and sample blanks were analysed in a blinded fashion for total Si content with their appropriately prepared standards all in a single batch.

Tibias

Tibias were digested and analysed using previously described methods [16]. One of the pair of tibias from each animal was cleaned of soft tissue, ligaments and tendons and then digested separately in 10 mL (females) or 12 mL (males) of an acid-water mixture consisting of: one part nitric acid (69% p.a. plus) and three parts UHP water. Samples were digested at 185°C (10 min ramp to 185°C and maintained at 185°C for 20 min) in acid-cleaned TFM vessels in a Milestone Ethos Plus microwave digestion system (Milestone Srl, Sorisole, Italy). This method gives a complete digestion of the bone samples (i.e. both the organic and inorganic components are digested). Digested samples were transferred to pre-weighed 13 mL PP tubes to determine final digest volumes and for ICP-OES analysis. Sample blanks were similarly prepared but without samples. Total Si analysis of the samples was carried out using sample-based standards (0-10 mg/L Si) prepared in aliquots of a pooled sample of the digested bones (1mL was removed from each of the digested bones for the final pool). Sample blanks were analysed with standards (0-10 mg/L Si) that were prepared in the acid-water mixture. All samples, including sample blanks, were analysed in a single batch. The digest samples and sample blanks were also analysed for Mn and Cu concentrations in a similar

fashion but further diluted in UHP water for the determination of other elements: 10-fold dilution for Zn, Fe and K concentrations; 100-fold dilution for Mg concentrations; and 1,000-fold dilution for Ca and P. Analysis was carried out using appropriately prepared sample-based standards in a blinded fashion.

Rodent feed

Samples (0.1 g; n=4) of the rat feed were digested with 5 mL of 1:1 mixture of 69% nitric acid and UHP water at 185°C (for 25 min, following a 15 min ramp to 185°C) in the Milestone Ethos Plus microwave digestion system. Ten mL UHP water was added to each sample at the end of the digestion run. Sample blanks (n=4) were similarly prepared. The digested samples and sample blanks were analysed in a blinded fashion for Si and other elements in a single batch with appropriately prepared multi-element sample-based standards (0-10 mg/L Si and other elements).

Bone quality measurements

The second of the pair of tibias collected from each animal at necropsy was cleaned as described above at the authors' laboratory and couriered, frozen on dry ice, from the authors' laboratory to the Laboratory of Pathophysiology, University of Antwerp, Belgium for micro-CT analysis. For analysis the bone samples were moistened in physiological saline and wrapped in parafilm to avoid reflection artefacts, as recommended for BMD measurement by the micro-CT manufacturer. Scans were performed on a SkyScan 1076 *in-vivo* X-ray micro-CT scanner (Aartselaar, Belgium) equipped with software version 3.2 (build 2) and a Hamamatsu Orca-HRF camera with pixel size of 11.74 µm. A scanning width of 35 µm and Image Pixel Size of 34.72 µm were used. Each tibia was scanned once and each scan consisted of 300 images: four images were collected and averaged at each 0.6° rotational step

over a 180° rotation of the bone. The resolution of the measurements was 35 µm. After reconstruction of the images, a number of bone quality (microarchitecture) parameters (measures) were derived, including: trabecular BMD (tBMD, g/cm^3), tissue volume (TV, µm³), bone volume (BV, µm³), bone volume fraction (BV/TV, %; the proportion of the total volume occupied by mineralised bone), total surface (TS, µm²), bone surface (BS, µm²), bone surface/volume ratio (BS/BV, 1/µm), bone surface density (BS/TV, 1/µm), trabecular thickness (Tb.Th, µm), trabecular separation (Tb.Sp, µm), trabecular number (Tb.N, 1/µm) and total porosity (Po(T), %). Trabecular BMD was determined from a calibration curve produced with phantom bones of specific BMD and due to shadow effects an error of ± 10 mg/cm^3 can be expected on these measurement. Trabecular separation and trabecular number were calculated separately, directly from the scans: i.e. they are not derived from each other. Total porosity is the sum of the open and closed pores and thus is a measure of the canalisation of the trabeculae. Samples were again analysed in a blinded fashion.

X-ray diffraction

One tibia from each of the groups of the female rats that had undergone micro-CT analysis underwent powder XRD analysis at the Institute for Materials Research, University of Leeds (UK). Prior to analysis the bones were crushed with a mortar and pestle, freeze dried and subjected to several solvent extractions (based on the method by Kim *et al*., (17)) to remove organic components from the bone matrix that could interfere with the XRD analysis: namely, 2:1 chloroform/methanol mixture (for 3 h at 4°C, ×3), ice-cold methanol rinses (×3), 6 M guanidine HCl in methanol (overnight at 4°C), ice cold methanol and ice cold ethanol rinses (×3). The bone samples were then dried overnight at 60°C in a dry oven before further grinding with a mortar and pestle and oxygen plasma ashing (10 W for 15 min, × 10 cycles in an Emitech K1050X Plasma Asher; Quorum Technologies Ltd, Ashford, UK). Fourier Infra-

Supplementary Data

Red analysis (Shimadzu IRPrestige-21 FT IR; Shimadzu, Kyoto, Japan) confirmed removal of much of the organic constituents in the bone matrix without any significant changes to the bone mineral. X-ray diffraction data were collected using a P'Analytical X'Pert MPD employing Cu $K\alpha_{1+2}$ radiation (Almelo, The Netherlands). The bone powders were sieved through a 300 micron gauze onto a low-background-Si substrate, and rotated during collection. The sample was scanned in the range $10 < 2\theta < 80$, in steps of 0.05°, for a total time of ca. 1 h. Programmable divergence and anti-scatter slits were used, with an irradiated area of 15 x 15 mm. XRD analysis was also carried out in a blinded fashion. Formal statistical comparisons were not intended as only one 'indicator' tibia per group was analysed, and this was only in case supplemental silicon had been highly loaded into bone yielding obvious spectral differences in bone quality.

Biomechanical testing

Tibias from female rats were subjected to three-point bending test at room temperature in a custom made loading device, integrated in a materials testing machine (Bose ElectroForce Test Bench LM1, Bose Corp, USA). The fibula was removed and tibia placed with the lateral surface facing down. Load was applied midway between two supports 1.00 mm distal and 14.0 mm proximal from the tibia-fibula junction. A stabilising preload was applied (ramped to 4 N at 0.2 N/s) followed by five conditioning cycles (oscillating between 4 N and 24 N at 0.05 Hz), dwell at 4 N for 5s, and finally loading until failure at a rate of 0.05 mm/s. It was verified *a posteriori* for all samples that the load of 40 N was located within the linear region of the force displacement ($F–\delta$) curve. From the $F–\delta$ curves the following parameters were calculated: stiffness (k; N/mm) was determined as the slope of the force–displacement curve at 40 N load; yield strength (F_y; N) was determined as the load where the tangent to the $F–\delta$

curve fell to 95% of the stiffness *k*; fracture load (F_{max}; N) was defined as the maximum load sustained by the structure. Analysis was carried out in a blinded fashion.

Supplementary Data

Supplementary Table 1. Element concentrations of the 2018 Teklad 18% Protein Rodent diet determined by analysis herein

Elements		Mean	SD
Ca	(mg/g)	8.391	1.053
P	(mg/g)	6.46	0.104
K	(mg/g)	7.572	0.052
Mg	(mg/g)	2.473	0.056
Si	(µg/g)	610.4	97.3
Fe	(µg/g)	195.9	22.0
Zn	(µg/g)	76.7	15.01
Mn	(µg/g)	85.56	8.43
Cu	(µg/g)	15.40	2.87
B	(µg/g)	8.852	2.653

Supplementary Data

Supplemental Table 2. Fasting serum element concentrations in the control and Si-supplemented groups after 12 weeks intervention

	Combined (Males & Females)			Female rats			Male rats		
Serum Elements	Group 1: Control (n=10)	Group 2: 115 mg Si/L (n=10)	Group 3: 575 mg Si/L (n=9)	Group 1: Control (n=5)	Group 2: 115 mg Si/L (n=5)	Group 3: 575 mg Si/L (n=4)	Group 1: Control (n=5)	Group 2: 115 mg Si/L (n=5)	Group 3: 575 mg Si/L (n=5)
Ca (mg/L)	85.8 (4.1)	87.6 (4.5)	88.1 (2.3)	86.2 (3.1)	90.4 (4.5)	88.2 (1.6)	85.4 (5.3)	84.7 (1.9)	88.1 (2.9)
Mg (mg/L)	22.7 (3.3)	23.2 (2.9)	21.5 (1.0)	22.8 (3.0)	24.9 (1.4)	21.3 (1.3)	22.6 (4.0)	21.6 (3.2)	21.8 (1.0)
K (mg/L)	323 (46)	315 (21)	325 (20)	294 (37)	315 (32)	315 (18)	361 (19)	315 (12)[d]	334 (19)
Zn (mg/L)	1.27 (0.21)	1.28 (0.10)	1.28 (0.08)	1.09 (0.10)	1.34 (0.10)[a]	1.22 (0.02)[b]	1.45 (0.09)	1.22 (0.06)[e]	1.33 (0.07)[f]
Cu (mg/L)	0.83 (0.25)	0.98 (0.38)	1.07 (0.26)	0.98 (0.23)	1.33 (0.19)[c]	1.12 (0.21)	0.65 (0.12)	0.70 (0.20)	1.02 (0.32)

Means (± SD) of samples collected at necropsy.

[a]*P*= 0.004, [b]*P*= 0.043, [c]*P*= 0.045, [d]*P*= 0.003, [e]*P*= 0.002, [f]*P*=0.047, [g]*P*= 0.032; vs. control (Student t-test).

Supplementary Data

Supplemental Table 3. Element concentrations in the tibia of rats in the control and Si-supplemented groups after 12 weeks intervention

	Combined (Males & Females)			Female rats			Male rats		
Tibia Elements	Group 1: Control (n=10)	Group 2: 115 mg Si/L (n=10)	Group 3: 575 mg Si/L (n=10)	Group 1: Control (n=5)	Group 2: 115 mg Si/L (n=5)	Group 3: 575 mg Si/L (n=5)	Group 1: Control (n=5)	Group 2: 115 mg Si/L (n=5)	Group 3: 575 mg Si/L (n=5)
Ca (mg/g)	153 (7)	156 (13)	158 (9)	158 (4)	164 (13)	165 (5)[c]	147 (6)	147 (5)	151 (5)
P (mg/g)	86.1 (7.3)	88.9 (9.3)	88.5 (5.7)	92.5 (3.5)	95.6 (8.8)	92.8 (3.7)	79.8 (2.9)	82.3 (2.5)	84.2 (3.5)
Mg (mg/g)	3.20 (0.22)	3.36 (0.31)	3.36 (0.12)[a]	3.35 (0.16)	3.55 (0.33)	3.39 (0.10)	3.05 (0.14)	3.17 (0.15)	3.33 (0.14)[e]
K (mg/g)	1.27 (0.29)	1.17 (0.38)	1.25 (0.29)	1.13 (0.36)	1.00 (0.48)	1.10 (36)	1.41 (0.11)	1.35 (0.13)	1.39 (0.11)
Zn (µg/g)	199 (19)	207 (32)	197 (17)	214 (15)	232 (27)	211 (7)	185 (10)	183 (11)	182 (8)
Fe (µg/g)	49.6 (12.6)	53.6 (6.6)	48.6 (10.3)	48.2 (8.9)	55.8 (7.1)	55.6 (9.5)	50.9 (16.6)	51.5 (6.2)	41.6 (5.1)
Cu (µg/g)	1.45 (0.14)	1.70 (0.31)[b]	1.56 (0.17)	1.52 (0.14)	1.60 (0.12)	1.47 (0.06)	1.38 (0.10)	1.81 (0.42)	1.64 (0.22)[f]
Mn (µg/g)	0.69 (0.10)	0.75 (0.09)	0.73 (0.08)	0.78 (0.06)	0.78 (0.05)	0.75 (0.04)	0.61 (0.05)	0.72 (0.11)	0.71 (0.11)
Ca:P	1.79 (0.09)	1.76 (0.06)	1.79 (0.03)	1.71 (0.04)	1.72 (0.06)	1.78 (0.03)[d]	1.85 (0.06)	1.80 (0.03)	1.79 (0.04)

Means (± SD) of samples collected at necropsy.

[a]*P*= 0.05, [b]*P*= 0.029, [c]*P*= 0.026, [d]*P*= 0.016, [e]*P*= 0.013, [f]*P*=0.043; vs. control (Student t-test).

Supplementary Data

Supplemental Table 4. Bone microarchitecture (quality) of rats in the control and Si-supplemented groups

	Combined (Males & Females)			Female rats			Male rats		
Tibia	Group 1: Control (n=10)	Group 2: 115 mg Si/L (n=10)	Group 3: 575mg Si/L (n=10)	Group 1: Control (n=5)	Group 2: 115 mg Si/L (n=5)	Group 3: 575 mg Si/L (n=5)	Group 1: Control (n=5)	Group 2: 115 mg Si/L (n=5)	Group 3: 575 mg Si/L (n=5)
tBMD (g/cm^3)	0.316 (0.155)	0.327 (0.136)	0.319 (0.155)	0.440 (0.115)	0.453 (0.016)	0.453 (0.088)	0.193 (0.053)	0.201 (0.043)	0.184 (0.028)
BV (mm^3)	1.67 (1.03)	1.69 (0.68)	1.96 (1.19)	2.10 (1.30)	2.12 (0.64)	2.63 (1.34)	1.24 (0.46)	1.26 (0.42)	1.29 (0.50)
TV (mm^3)	32.8 (14.1)	32.5 (12.4)	35.2 (12.9)	20.5 (3.4)	21.9 (5.9)	23.3 (2.6)	45.0 (7.7)	43.0 (5.8)	47.0 (4.2)
BS (mm^2)	88.4 (44.6)	91.6 (36.7)	100 (52.4)	107 (52)	112 (37)	130 (58)	69.8 (29.6)	71.6 (26.3)	70.4 (24.3)
TS (mm^2)	78.0 (20.2)	77.5 (20.1)	80.9 (17.6)	60.2 (5.9)	60.7 (9.3)	64.4 (2.3)	95.9 (9.1)	94.3 (10.7)	97.5 (3.2)
TbTh (mm)	0.115 (0.010)	0.117 (0.009)	0.118 (10.3)	0.113 (0.011)	0.117 (0.007)	0.121 (0.007)	0.117 (0.009)	0.116 (0.011)	0.115 (0.010)
TbSp (mm)	0.777 (0.475)	0.697 (0.397)	0.740 (0.382)	0.397(0.110)	0.379 (0.044)	0.398 (0.137)	1.16 (0.37)	1.02 (0.32)	1.08 (0.13)
TbN (1/mm)	0.555 (0.455)	0.538 (0.315)	0.563 (0.411)	0.876 (0.451)	0.825 (0.119)	0.893 (0.322)	0.234 (0.072)	0.251 (0.064)	0.233 (0.070)
PoT (%)	93.5 (6.0)	93.7 (3.8)	93.2 (5.2)	89.7 (6.6)	90.3 (1.8)	89.1 (4.3)	97.3 (0.7)	97.1 (0.6)	97.3 (1.0)

Means (± SD) of samples collected at necropsy.

Supplementary Data

Supplemental Table 5. Biomechanical data of rats in the control and Si-supplemented groups

	Combined (Males & Females)			Female rats			Male rats		
Tibia	Group 1: Control (n=7)	Group 2: 115 mg Si/L (n=10)	Group 3: 575 mg Si/L (n=10)	Group 1: Control (n=5)	Group 2: 115 mg Si/L (n=5)	Group 3: 575 mg Si/L (n=5)	Group 1: Control (n=2)[a]	Group 2: 115 mg Si/L (n=5)	Group 3: 575 mg Si/L (n=4)
k (N/mm)	398 (73)	405 (98)	412 (89)	365 (30)	340 (57)	348 (46)	478	470 (89)	491 (55)
Fy (N)	101 (9.3)	101 (25)	111 (31)	99 (5.3)	89 (18)	87 (6.8)	107	114 (27)	143 (14)
Fmax (N)[d]	125 (25)	126 (24)[b]		120 (8.6)	113 (13)	114 (8.5)	137	156[c]	

Means (± SD) of samples collected at necropsy.

[a]Data collected from three of the bones was unreliable due to vibration of the samples during data collection. The measurement protocol was modified for remainder of male rat bones.

[b]N=7, [c]N=2.

[d]Majority of bones from the male rats did not break with the maximum load of 180 N, allowed by testing apparatus used.

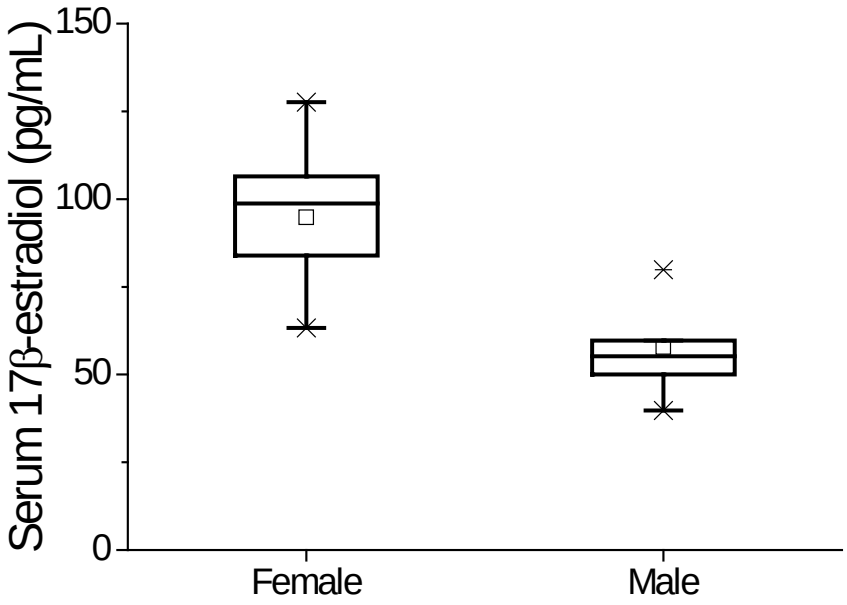
Supplementary Data

Supplemental Table 6. Pearson correlations for serum osteocalcin and serum 17β-estradiol[a]

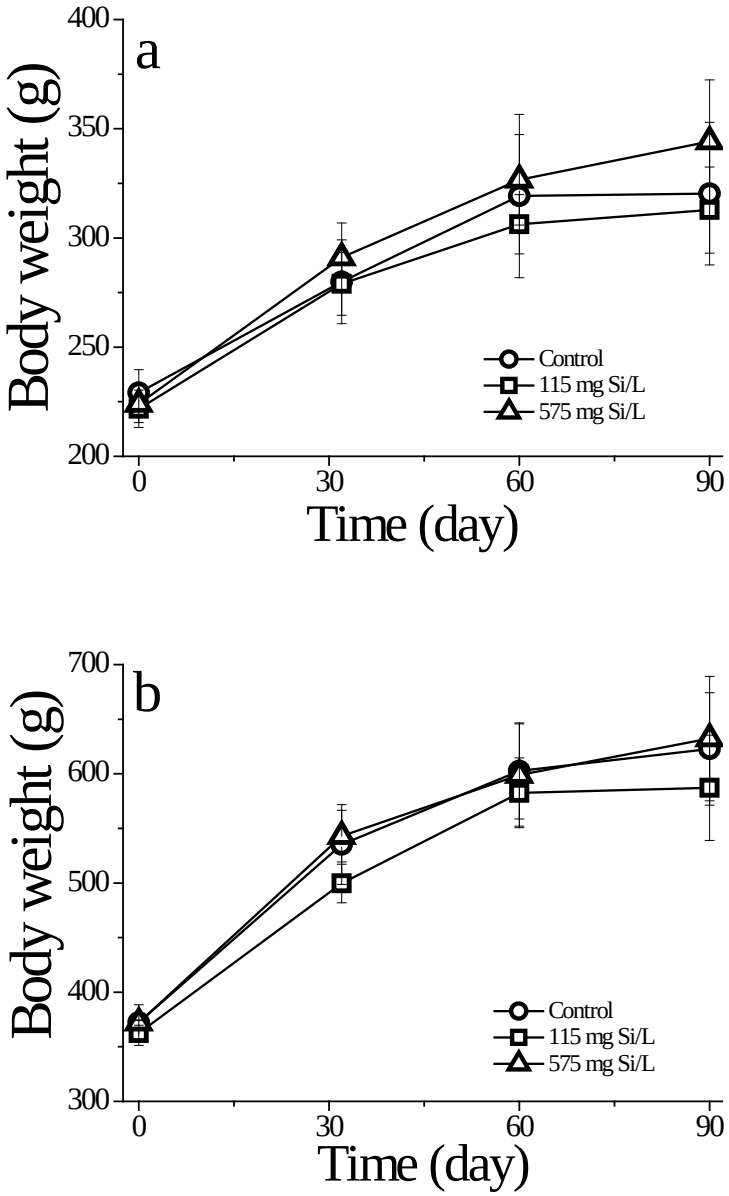
	Combined		Male rats		Female rats	
	r	p	r	p	r	p
Serum Osteocalcin *vs.*						
Serum Si	-0.57	0.004	-0.72	0.008		
Tibia P			-0.61	0.034		
Tibia Mg			-0.77	0.003	-0.62	0.031
Fy (yield strength)	-0.62	0.003	-0.69	0.042		
Serum 17β-estradiol *vs.*						
tBMD						
TV	0.68	0.001				
BV/TV	-0.77	0.0001				
TS	0.47	0.043				
BS/BV	-0.80	0.0005				
TbSp	0.56	0.013				
TbN	-0.68	0.002				
TbTh	0.54	0.016				
PoT					-0.67	0.05
Serum osteocalcin	-0.47	0.043				
Ca:P ratio			0.71	0.033		
Tibia P	-0.58	0.010				
Serum K	0.47	0.044				
Tibia Zn	-0.52	0.023				
Tibia length	0.60	0.007				
k (stiffness)	-0.74	0.001				
Fy (yield strength)	-0.57	0.018				
Body weight	-0.65	0.005	-0.88	0.004		
Body weight gain	-0.80	0.00004			-0.96	0.00005
	-0.78	0.00008			-0.95	0.00008

[a]Only statistically significant correlations are shown.

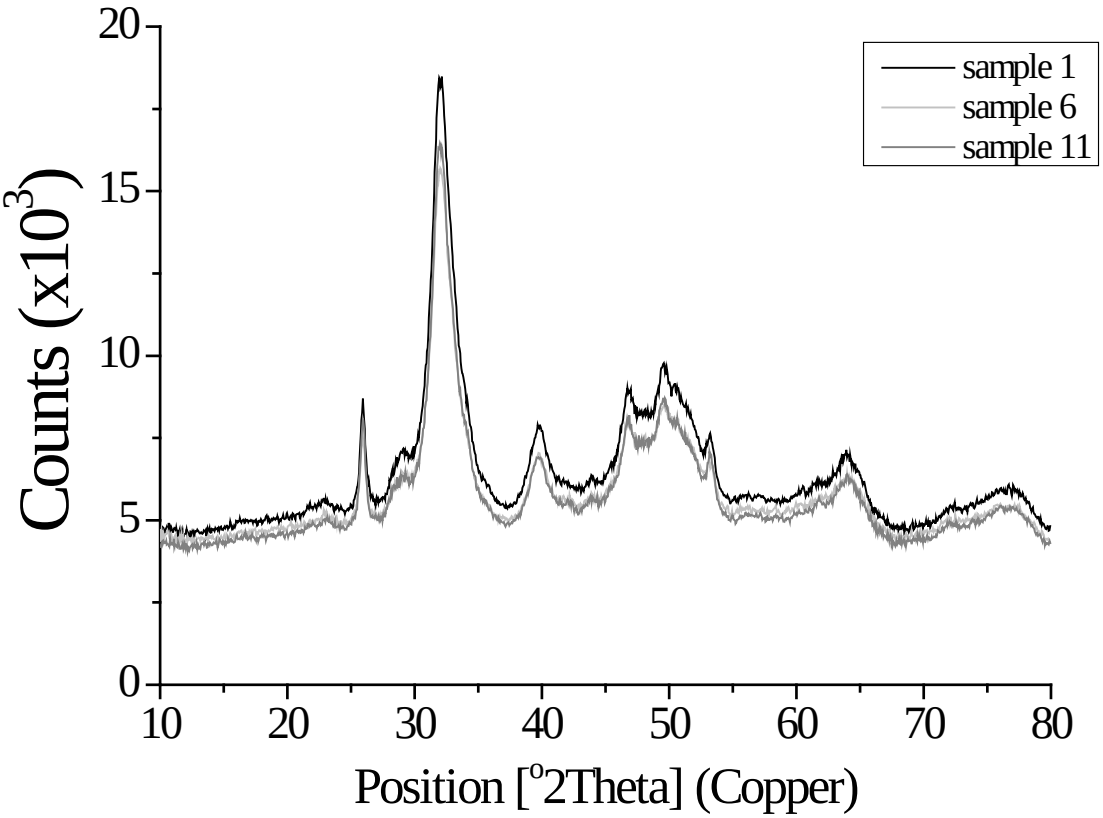
Supplementary Data



Supplemental Figure 1. 17β-estradiol concentrations of the fasting serum, collected at necropsy, of the female (n=9) and male (n=10) rats after 12 weeks supplementation with Si (medium or high dose, or vehicle). Data is shown as box-plots where the horizontal lines indicate the 5th, 25th, 50th (i.e. median), 75th and 95th percentiles, the open square shows the mean and the crosses the minimum and maximum values. Serum 17β-estradiol concentration was on average 1.7 fold higher in the female rats compared to the male rats ($p = 0.0002$, independent sample t-test).



Supplemental Figure 2. Body weights of female (**a**) and male (**b**) rats over the 90 days of intervention with the diluent control, 115 mg Si/L and 575 mg Si/L in their drinking water. Data are means ± SD of 5 rats at each time points.



	Lattice parameters[a]	
Samples	*a*	*c*
1	9.445	6.886
6	9.449	6.888
11	9.448	6.884

[a] ±0.002

Supplemental Figure 3. X-ray diffraction (XRD) analysis of the mineral phase of the tibias of female rats after 12 weeks intervention with diluent control (sample 1), 115 mg Si/L (sample 6) and 575 mg Si/L (sample 11) in their drinking water. The lattice parameters of the three samples are tabulated.